

IN THE SPECIFICATION:

Please replace the sequence listing at pages 24-28 with the substitute sequence listing submitted concurrently herewith.

Please amend the paragraph beginning at page 2, line 19, as follows:

Recently, a mutant of GFP into which the mutations of Y66H and Y145F were introduced and which had different wavelength characteristics (it is also referred to as “Mutant,” and its amino acid sequence (SEQ ID No. 15) is described below [with the above-mentioned mutations shown as underlined]) was developed. This is referred to as “BFP (Blue Fluorescent Protein),” because it emits blue fluorescence by UV excitation. (R. Heim et al. Curr. Biol. 6: 178-182 (1996); R. Heim et al. Proc. Natl. Acad. Sci. USA 91: 12501-04 (1994).) In the present specification, the term “BFP protein” refers to a protein that emits blue fluorescence when excited by ultraviolet-blue light and that, then, does not require an energy source such as a special substrate or ATP. However, such BFP had a problem in that it experienced severe fading as compared to GFP and was difficult to be observed under the microscope or the like. As used herein to designate mutation, the position of the mutation is expressed by a specific amino acid number in the sequence of the above-mentioned wild type; the amino acid prior to its mutation is described preceding the number and the mutated amino acid is to be described following the number.

Please amend the line at page 3, line 33, as follows:

Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys (SEQ ID NO: 15).

Please amend paragraph at page 4, lines 7-10 as follows:

In view of the above-mentioned problems, the present inventors [~~made extensive researches~~] performed extensive research and succeeded in the discovery of novel GFPs and BFPs that are free from such problems by introducing certain mutations into specific positions of the amino acid sequence for GFP or BFP, thus accomplishing this invention.

Please amend the heading at page 7, line 29 as follows:

DESCRIPTION OF THE ~~[PREFERRED]~~ PREFERRED EMBODIMENTS

Please amend the paragraph beginning at page 14, line 18, as follows:

In this invention, a DNA portion encoding GFP of pGFP-C1 (available from Clontech Inc.) was replaced by a DNA of GFP derived from phGFP-S65T (available from Clontech Inc.), which served as a basic plasmid (hereinafter referred to as "phGFP(101)-C1"). The vector is meant for expression in mammalian cells and its full base sequence including the vector part is known in the art. The corresponding amino acid sequence (SEQ ID No. 14) is set forth below.

Please amend the paragraph at page 11, lines 7-12 as follows:

Moreover, methods for randomly introducing mutations are not particularly limited, and Mutagenic PCR as described below can preferably be used in this invention. The Mutagenic PCR can be carried out according to methods known in the art. (C.W. Dieffenback, ed. PCR PRIMER, A Laboratory Manual (Cold Spring Harbor Laboratory Press) [~~(1955)~~] (1995) pp. 583-588.) Concretely, the following conditions were employed in the examples.

Please amend paragraph at Page 11, lines 13-21 as follows:

About 50 ng of Plasmid BlueBFP (201) was added to [~~10xmutagenic~~] 10 × mutagenic PCR buffer (70 mM MgCl₂, 500 mM KCl, and 100 mM Tris-HCl, pH 8.3 at 25 °C; 0.1% (w/v) gelatin) 10 µl, [~~10xdNTP~~] 10 × dNTP (2 mM dGTP, 2 mM dATP, 10 mM dCTP, and 10 mM dTTP) 10 µl, 10 mpol/µl primer (23mer M13Universal primer and M13Reverse primer) 3µl, and H₂O 62µl, and mixed.. Subsequently, 10 µl of 5mM MnCl₂ was added and mixed, and 1µl of Taq Polymerase (Takara) was added to conduct PCR (PC-700 available from ASTEC Inc. was used). The PCR was conducted in three tubes under the following conditions: 25 cycles at 94 °C for 1 min, 30 cycles at 45 °C for 1 min, and 35 cycles at 72 °C for 1 min, respectively.

Please amend the paragraph beginning at page 12, line 10, as follows:

The site-directed mutation introduction methods are not particularly limited, and for example, the protocol for a Quick Change Kit from Stratagene Inc. was followed. The

oligonucleotides shown in Table 2 below (SEQ ID Nos. 2-13, respectively) were used as primers and the plasmid (about 0.03 µg) obtained by subcloning GFP or [BFPcDNA] BFP cDNA into the HindIII site of a pUC18 or pQE30 vector was used as a template. The concrete PCR conditions are preferably as follows: 16 cycles at 95 °C for 30 sec, 55 °C for 1 min, and 68 °C for 10 min.

Please amend Table 4 on Pages 14-15 as follows:

TABLE 4

GFP						
101	none					
103	Phe64Leu					
104			Val1163Ala		Ser175Gly	
105			Phe64Leu,	Val1163Ala,	Ser175Gly	

BFP (as for BFP, the two mutations, [Y66H] <u>Tyr66His (Y66H)</u> and [Y145F] <u>Tyr145Phe (Y145F)</u> , have been introduced into the sequence for GFP which serves as a basis)						
201	Y66H,	Y145F:				
202	Y66H,	Y145F:	Phe64Leu,	Leu236Arg		
203	Y66H,	Y145F:	Phe64Leu			
204	Y66H,	Y145F:			Val1163Ala,	Ser175Gly
205	Y66H,	Y145F:	Phe64Leu,	Val1163Ala,	Ser175Gly,	Leu236Arg

Please amend paragraph at Page 16, lines 15-31 as follows:

Unless otherwise so stated, CHO-K1 cells were grown in a F12+10% FBS medium in 5% CO₂ at 37 °C. The cells [~~(1×10⁵)~~](1 × 10⁵) were inoculated into a 6-cm dish, and on the following day, their transfection was conducted in two dishes as a pair by the calcium phosphate method. (C. Chen and H. Okayama Mol. Cell. Biol. 7: 2745-2752 (1987).) After transfection, the one dish was incubated at 37 °C and the other at 30 °C for 24 h. The transfected CHO cells were washed with [~~1×PBS (-)~~] 1 × PBS (-) three times, and they were dissolved in 1 ml of 10 [~~mM Tris-HCl~~] mM Tris-HCl (pH 7.4) containing 1% Triton X-100 and recovered in an Eppendorf tube. A supernatant (0.5 ml) from centrifugation at 3,000 rpm for 5 min was diluted 4-fold with the same buffer and fluorescence measurement was performed. Here, a pUcD2SRαMCS vector (empty vector) was transfected and used as a blank. A Hitachi F-2000 type fluorophotometer was used in the fluorescence measurement. In the measurement of GFPs, fluorescence was scanned between 460 nm and 600 nm at an excitation wavelength of 460 nm to measure the maximal value in the vicinity of the fluorescence wavelength of 510 nm. In the measurement of BFPs, fluorescence was scanned between 360 nm and 500 nm at an excitation wavelength of 360 nm to measure the maximal value in the vicinity of the fluorescence wavelength of 445 n.

Please amend paragraph at Page 17, lines 2-20 as follows:

The CHO cells were transfected with pUcD2SRαMCS (empty vector)(T. Tsukamoto et al. Nature. Genet. 11: 395-401 (1995)), phGFP(101)-Cl, phGFP(105)-Cl, phBFP(201)-CL, and phBFP(205)-Cl, respectively and grown at 37 °C and at 30 °C. Employing a sample prior to dilution as used in the fluorescence measurement previously described (8 μl), SDS-PAGE was performed on a 12% gel. With the use of a Horizonblot (ATTO Inc.), transfer was conducted onto a nitrocellulose membrane (Millipore Inc., HAHY394FO) under the conditions of 2 mA and 90 min per cm². After the membrane was taken out and washed with [~~1×~~PBS] 1 × PBS, it was immersed in 1% skim milk/PBS and shaken at room temperature for 30 min. After the membrane was washed with [~~1×~~PBS] 1 × PBS, it was immersed in 0.1% skim milk/PBS containing an anti-GFP antibody (Clonotech Inc.) that had been diluted 2,000-fold and shaken at 4 °C overnight. The membrane was washed with [~~1×~~PBS] 1 × PBS for 5 min, and then with TPBS (0.05% Trion X-100/PBS) for 15 min three times. The membrane was immersed in 0.1% skim milk/PBS containing an anti-rabbit IgG antibody labeled with HRP (Amersham Inc.) that had been diluted 1,000-fold, and shaken at 4 °C for 1 h. The membrane was washed with [~~1×~~PBS] 1 × PBS for 5 min, and then with TPBS (0.05% Trion X-100/PBS) for 15 min three times. The membrane was reacted with a chemiluminescence reagent (Amersham Inc. ECL) for 1 min, and then, was exposed to an X-ray film for 2 min.